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Acclimation of Chlamydomonas to changing carbon availability

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Abstract
Aquatic organisms, including *Chlamydomonas reinhardtii*, are faced with a variable supply of dissolved inorganic carbon (Ci). Accordingly, *C. reinhardtii* has the ability to acclimate to the changing Ci supply through a variety of responses, including induction of a CO2 concentrating mechanism (CCM) when Ci is limiting. The CCM uses active Ci uptake to accumulate a high internal concentration of bicarbonate, which is dehydrated by a specific thylakoid carbonic anhydrase to supply CO2, the substrate used in photosynthesis. In addition to the changes demonstrably related to the function of the CCM, *C. reinhardtii* exhibits several other acclimation responses to limiting Ci, such as changes in cellular organization and induction or upregulation of several genes. A key area currently under investigation is how *C. reinhardtii* cells recognize the change in Ci or CO2 concentration, and transduce that signal into needed gene expression changes. Mutational analyses are proving very useful for learning more about the CCM and about the acclimation response to changes in Ci availability. Cloning of the gene disrupted in *cia*5, a mutant apparently unable to acclimate to limiting Ci, has opened opportunities for more rapid progress in understanding the signal transduction pathway. The *Cia5* gene appears to encode a transcription factor that may control, either directly or indirectly, much of the gene expression responses to limiting Ci in *C. reinhardtii*. Several additional new mutants with potential defects in the signal transduction pathway have been isolated, including three new alleles of *cia5*.

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Acclimation of *Chlamydomonas* to changing carbon availability

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Abstract. Aquatic organisms, including *Chlamydomonas reinhardtii*, are faced with a variable supply of dissolved inorganic carbon (Ci). Accordingly, *C. reinhardtii* has the ability to acclimate to the changing Ci supply through a variety of responses, including induction of a CO_2 concentrating mechanism (CCM) when Ci is limiting. The CCM uses active Ci uptake to accumulate a high internal concentration of bicarbonate, which is dehydrated by a specific thylakoid carbonic anhydrase to supply CO_2, the substrate used in photosynthesis. In addition to the changes demonstrably related to the function of the CCM, *C. reinhardtii* exhibits several other acclimation responses to limiting Ci, such as changes in cellular organization and induction or upregulation of several genes. A key area currently under investigation is how *C. reinhardtii* cells recognize the change in Ci or CO_2 concentration, and transduce that signal into needed gene expression changes. Mutational analyses are proving very useful for learning more about the CCM and about the acclimation response to changes in Ci availability. Cloning of the gene disrupted in *cia5*, a mutant apparently unable to acclimate to limiting Ci, has opened opportunities for more rapid progress in understanding the signal transduction pathway. The *Cia5* gene appears to encode a transcription factor that may control, either directly or indirectly, much of the gene expression responses to limiting Ci in *C. reinhardtii*. Several additional new mutants with potential defects in the signal transduction pathway have been isolated, including three new alleles of *cia5*.

Introduction

C. reinhardtii and other aquatic or soil-borne photosynthetic organisms can be exposed to dramatic long-term and short-term changes in the supply of dissolved Ci because of variability in the sediment or soil respiration and the very slow diffusion of CO_2 (and Ci) in water. To survive such conditions, *C. reinhardtii* and other aquatic photosynthetic organisms are able to acclimate rapidly to changes in Ci supply. Fundamental to this acclimation is a mechanism to concentrate Ci internally when it is limiting (CO_2 concentrations at or below equilibrium with air). The most extensively studied of these mechanisms are the CCMs of cyanobacteria and microalgae (reviewed in Spalding 1998; Kaplan and Reinhold 1999; Badger and Spalding 2000). Historically, interest in the microalgal CCM centred on its potential for improving the efficiency of CO_2 assimilation under ambient CO_2 concentrations, but interest has increased recently in the mechanisms by which microalgae recognize and acclimate to changing CO_2 concentrations, as potential models for understanding how terrestrial plants might respond to increasing atmospheric CO_2.

The CO_2 concentrating mechanism

Among limiting-CO_2 acclimation responses, the CCM has received the most attention because of its effect on photosynthetic characteristics. Essential components of the microalgal CCM include, at least, active Ci uptake for intracellular bicarbonate accumulation, and internal carbonic anhydrase (CA) for dehydration of the accumulated bicarbonate to supply CO_2 to Rubisco (Badger *et al.* 1980; Spalding *et al.* 1983*a*, *b*; Moroney *et al.* 1985, 1987*b*).

In spite of substantial research on Ci transport and the isolation of a *C. reinhardtii* mutant (*pmp1-1*; Table 1) apparently lacking active Ci uptake (Spalding *et al.* 1983*b*), our understanding of Ci uptake in microalgae remains unclear, both in terms of location and substrate specificity. Carbon isotope disequilibrium studies established that the major flux of Ci into *C. reinhardtii* occurs through uptake of CO_2 via an active process (Marcus *et al.* 1984; Sültemeyer

Abbreviations used: CA, carbonic anhydrase; CCM, CO_2 concentrating mechanism; Ci, inorganic carbon; HCR, high-CO_2 requiring.

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et al. 1989; Badger *et al.* 1994; Palmqvist *et al.* 1994), although the data cannot distinguish between active CO_2 transport and CO_2 diffusion into the cell along a gradient maintained by (for example) either active Ci transport into the chloroplast or active CO_2 hydration to bicarbonate, as suggested for cyanobacterial CO_2 uptake (Kaplan and Reinhold 1999; Shibata *et al.* 2001). Active Ci uptake into isolated chloroplasts (Moroney *et al.* 1987*a*; Sültemeyer *et al.* 1988) and bicarbonate transport into *C. reinhardtii* cells (Sültemeyer *et al.* 1989; Badger *et al.* 1994; Palmqvist *et al.* 1994) has been demonstrated. Thus, *C. reinhardtii* apparently can use both of the Ci species that are predominant in the aquatic environment (CO_2 and HCO_3^-), but prefers CO_2, and although bicarbonate must be transported across the plasmalemma, it is not clear what species of Ci is transported into the chloroplast, nor whether CO_2 is directly transported at all.

The essential internal CA, as defined by mutations at the CA1 locus (Spalding *et al.* 1983*a*; Moroney *et al.* 1986; Suzuki and Spalding 1989), was identified as a thylakoid lumen CA (ctCA1; Table 1) encoded by *Cah3* (Funke *et al.* 1997; Karlsson *et al.* 1998). Mutants defective in *Cah3* overaccumulate Ci, but are CO_2 limited in photosynthesis, a situation that can be mimicked or phenocopied by treatment with the membrane-permeant CA inhibitor, ethoxyzolamide (Spalding *et al.* 1983*a*; Moroney *et al.* 1987*b*; Suzuki and Spalding 1989; Park *et al.* 1999). Since the overaccumulated Ci is unavailable to Rubisco, which uses CO_2 as its substrate, mutants with lesions at the CA1 locus must accumulate Ci in the form of bicarbonate. These results suggest that bicarbonate is actively accumulated in the chloroplast, and that ctCA1 may be required for rapid dehydration of this accumulated bicarbonate to supply Rubisco with CO_2 at a physiological rate. However, recent results of van Hunnik and Sültemeyer (2002) and Villarejo *et al.* (A. Villarejo, pers. comm.) cast doubt on whether the ctCA1 is involved directly in the dehydration of accumulated bicarbonate.

In spite of the clear requirement for an internal CA in the CCM of *C. reinhardtii*, most of the CA activity in cells acclimated to limiting-CO_2 is from periplasmic CA, the involvement of which in the CCM is controversial (Moroney *et al.* 1985; Williams and Turpin 1987; Van and Spalding 1999). There are two periplasmic CA isozymes (Fujiwara *et al.* 1990; Fukuzawa *et al.* 1990; Rawat and Moroney 1991), the highly expressed pCA1 (*Cah1* gene product) induced by limiting CO_2, and pCA2 (*Cah2* gene product) which is repressed in CO_2-limited cells and expressed very weakly only in elevated CO_2. The role of pCA2 is unknown, but pCA1 may facilitate the use of external bicarbonate at neutral and alkaline pH (Moroney *et al.* 1985). Although confirming that pCA1 provides some benefit at low CO_2 concentrations, evidence from a *Cah1* null mutant (*cah1-1*;

Table 1. *Chlamydomonas reinhardtii* mutants of the CCM, CO_2 acclimation response, or related processes
WT, similar to wild-type growth; LHCR, growth much slower than WT; SHCR, no growth; LC, low CO_2 (~300–500 μL L^{-1} CO_2); VLC, very low CO_2 (~50–100 μL L^{-1} CO_2); ND, not determined

Locus	Allele (synonym)	Gene (synonym)	Protein (synonym)	Phenotype	References
CA1	*ca-1* (*ca-1-12-1C*)	*Cah3*	CtCA1 Thylakoid lumen α-CA	LHRC-LC; SHCR-VLC Ci overaccumulation	Funke *et al.* 1997; Karlsson *et al.* 1998; Spalding *et al.* 1983*a*
	ca1-2 (*cia-1*)				Moroney *et al.* 1986
	ca1-3 (*cia-2*)				Moroney *et al.* 1986
	ca1-4 (*cia-3*)				Moroney *et al.* 1986
	ca1-5 (*ca-1-18-6A*)				Suzuki and Spalding 1989
	ca1-6 (*ca-1-18-7C*)				Suzuki and Spalding 1989
CAH1	*cah1-1*	*Cah1*	pCA1 Major periplasmic CA	WT-LC; WT-VLC	Fukuzawa *et al.* 1990; Van and Spalding 1999
CIA5	*cia5-1*	*Cia5* (*Ccm1*)	Cia5 (Ccm1) Putative transcription factor	LHCR-LC; SHCR-VLC No limiting CO_2 acclimation	Fukuzawa *et al.* 2001; Moroney *et al.* 1989; Spalding *et al.* 1991; Xiang *et al.* 2001
	cia5-2				Fukuzawa *et al.* 2001
	cia5-3				Van *et al.* 2001
	cia5-4				Van *et al.* 2001
GDH1	*gdh1-1*	*Gdh1*	CRGDH Glycolate DH	SHCR-LC; SHCR-VLC No glycolate DH activity	Y. Nakamura, S. Kanakagiri, K. Van and M. Spalding, unpublished results
PGP1	*pgp1-1*	*Pgp1*	PGP1 P-glycolate phosphatase	SHCR-LC; SHCR-VLC No P-glycolate phosphatase activity	Suzuki *et al.* 1990; Mamedov *et al.* 2001
PMP1	*pmp1-1*	ND	ND	SHCR-LC; WT-VLC Lacks Ci transport in LC	Spalding *et al.* 1983*b*

Table 1) also demonstrated that its activity is not essential for growth in low CO_2 (Van and Spalding 1999), calling into question whether it is an essential component of the CCM.

CO_2 regulated changes in cell organization

Acclimation to limiting CO_2 involves substantial structural changes in *C. reinhardtii* cells, including an increase in the pyrenoid starch sheath (Kuchitsu *et al.* 1988; Ramazanov *et al.* 1994) and changes in mitochondrial distribution (Geraghty and Spalding 1996). Although suggested at one time to be functionally important as a CO_2 diffusion barrier (Badger and Price 1994), the pyrenoid starch sheath is apparently not required for efficient operation of the CCM (Villarejo *et al.* 1996*a*). The changes in mitochondrial distribution, moving from a central position to a peripheral position between the chloroplast envelope and the plasmalemma (Geraghty and Spalding 1996), are quite intriguing, because upregulation of several genes encoding mitochondrial proteins (Eriksson *et al.* 1996; Geraghty and Spalding 1996; Y. Nakamura, S. Kanakagiri, K. Van and M. Spalding, unpublished results) is coincident with mitochondrial relocation. These observations argue that mitochondria may play an important role in acclimation to limiting CO_2.

CO_2 regulated gene expression

Coincident with induction of a functional CCM, expression of several genes is induced *de novo* (Table 2), including *Cah1*, *Mca1* and *Mca2* (encoding identical 21 kDa mitochondrial β-CAs; Eriksson *et al.* 1996; Geraghty and Spalding 1996) and *Ccp1* and *Ccp2* (encoding nearly identical 36 kDa chloroplast inner-envelope proteins; Ramazanov *et al.* 1993; Chen *et al.* 1997). Several other genes have also been identified as being either induced or upregulated by limiting CO_2, including *Lci1* (Burow *et al.* 1996), *Att1* (Chen *et al.* 1996), cyclophilin (Somanchi and Moroney 1999), *Pgp1* (Mamedov *et al.* 2001) and *Gdh1* (Y. Nakamura, S. Kanakagiri, K. Van and M. Spalding, unpublished results). Since expression of these genes appears to be correlated and regulated by message abundance, their transcription may be co-ordinately regulated, at least in part, by the same mechanism. Interestingly, *Cah2* appears to be regulated in a manner inverse to the others (Fujiwara *et al.* 1990; Rawat and Moroney 1991). In contrast to these apparently transcriptionally regulated genes, the transiently decreased expression of both small and large subunits of Rubisco is controlled at the translational level (Winder *et al.* 1992). Other than Rubisco and the photorespiratory enzymes encoded by *Pgp1* and *Gdh1*, it is not clear what role, if any, the products of any of these genes might play in acclimation to limiting CO_2.

Little research has been devoted to the regulation of specific CCM components in microalgae. One essential component, ctCA1, is expressed constitutively in *C. reinhardtii* (Karlsson *et al.* 1998), while the limited data

Table 2. *Chlamydomonas reinhardtii* genes and proteins regulated by CO_2 concentration

Direction of regulation in limiting CO_2 is indicated. Some molecular sizes are predicted from the gene coding for the protein prior to any processing. Both subunits of pCA1 and pCA2 are processed from a single polyprotein translation product. ND, not determined. Data were compiled from: Coleman and Grossman 1984; Goldschmidt-Clermont and Rahire 1986; Fujiwara *et al.* 1990; Fukuzawa *et al.* 1990; Geraghty *et al.* 1990; Marek and Spalding 1991; McKay and Gibbs 1991; Rawat and Moroney 1991; Ramazanov and Cardenas 1992, 1994; Winder *et al.* 1992; Ishida *et al.* 1993; Ramazanov *et al.* 1993; Burow *et al.* 1996; Chen and Silflow 1996; Chen *et al.* 1996, 1997; Eriksson *et al.* 1996; Geraghty and Spalding 1996; Mamedov *et al.* 2001; Y. Nakamura, S. Kanakagiri, K. Van and M. Spalding, unpublished results. Table modified from Spalding (1998)

Gene (synonym)	Protein (synonyms)	Size (kDa)	Identification	Intracellular location
Upregulated				
Att1	AlaAT	58	Alanine:α-KG aminotransferase	ND
Cah1	pCA1	37, 4	α-type CA	Periplasmic space
Ccp1 (*LIP36G1*)	Ccp1 (LIP–36)	36	Transmembrane protein	Chloroplast inner envelope
Ccp2 (*LIP36G2*)	Ccp2 (LIP–36)	36	Transmembrane protein	Chloroplast inner envelope
Lci1	Lci1	21	Transmembrane protein	ND
Mca1 (*β-CA1*)	mtCA1 (β-CA1, LIP–21)	21	β-type CA	Mitochondria
Mca2 (*β-CA2*)	mtCA2 (β-CA2, LIP–21)	21	β-type CA	Mitochondria
Gdh1	CRGDH	119	Glycolate DH	Mitochondria (?)
Gs2	GS2	42	Glutamine synthetase	Chloroplast stroma
Transiently upregulated				
Pgp1	PGPase	32	P-glycolate phosphatase	Chloroplast stroma
Downregulated				
Cah2	pCA2	39, 4.5	α-type CA	Periplasmic space
Transiently downregulated				
rbcL	rbcL	55	Rubisco large subunit	Pyrenoid
RbcS1	RbcS1	16	Rubisco small subunit	Pyrenoid
RbcS2	RbcS2	16	Rubisco small subunit	Pyrenoid

available for active Ci uptake indicate that little or no bicarbonate transport or active CO_2 uptake occurs in cells grown in elevated CO_2. Thus, it is likely that any Ci transporters are either induced or activated in response to CO_2 limitation, but no Ci transporters have been identified so far. Interestingly, while the *pmp1-1* mutant (Table 1) was thought to be completely devoid of Ci transport, the recently discovered ability of this mutant to grow well in very low CO_2 (Fig. 1) clearly indicates it has a functional CCM under these conditions. Note that the apparently vigorous growth of *cia5* under low-CO_2 conditions (Fig. 1) is anomalous. In many similar growth tests, *cia5* typically grew only slowly in low CO_2, which is in agreement with its original description (Moroney *et al*. 1989), and with its description in Tables 1 and 3, as having a leaky high-CO_2 requiring (HCR) phenotype in low-CO_2 conditions.

The limiting-CO_2 signal and signal transduction

Acclimation of *C. reinhardtii* to changes in CO_2 concentration clearly is under environmental control, but it is not known whether the cells sense a change in the external CO_2 availability directly as CO_2 concentration, or indirectly via its effect on, for example, carbohydrate metabolism. Acclimation often has been studied using expression of *Cah1* as a reporter by monitoring CA activity, protein (pCA1) abundance, or mRNA abundance. Reports that induction (or de-repression) of periplasmic CA by limiting-CO_2 concentrations required light, was blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the light, and did not occur in non-photosynthetic mutants, argue for a photosynthetic activity requirement (Spalding and Ogren 1982; Spencer *et al.* 1983; Dionisio *et al.* 1989*a*, *b*; Dionisio-Sese *et al.* 1990; Villarejo *et al.* 1996*b*). Induction of pCA1 was

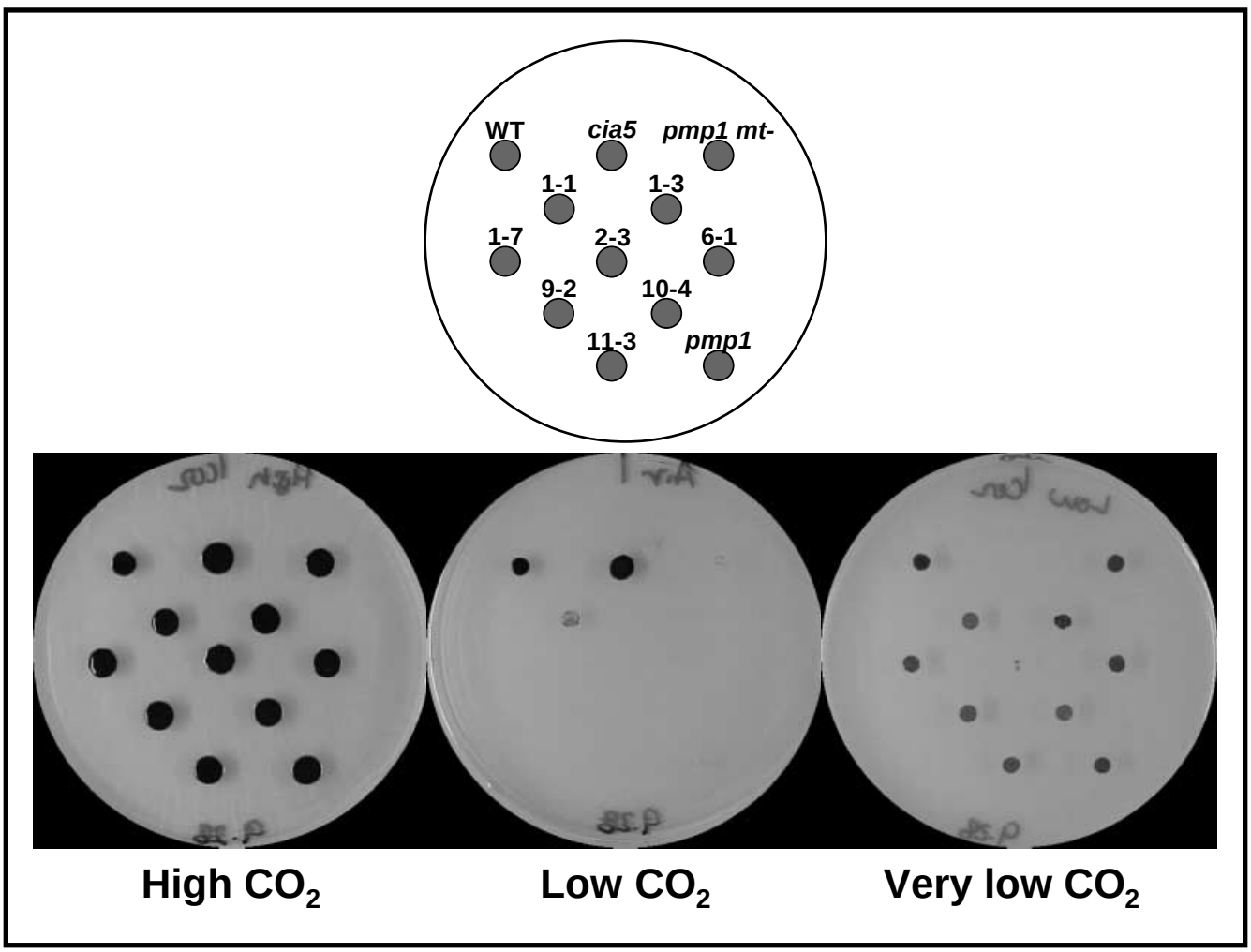

Fig. 1. Spot tests for growth response to different CO_2 concentrations for wild-type (WT) strain (CC400), previously described regulatory mutant CC2702 (*cia5-1*), previously described HCR mutant CC1860 (*pmp1-1*), and several *pmp1-1*-containing derivatives of CC1860, either from crosses with CC513 (1-1, 1-3, 1-7, 2-3, 6-1, 9-2, 10-4, 11-3) or with CC125 (*pmp1-1* mt-). Plates were kept either at high CO_2 (5% CO_2), at low CO_2 (normal air), or at very low CO_2 (50–100 μL L^{-1}) for 10 d.

also reportedly decreased by decreased O_2 tension and by photorespiratory inhibitors, suggesting that a photorespiratory metabolite might signal CO_2 limitation (Spalding and Ogren 1982; Ramazanov and Cardenas 1992; Villarejo *et al.* 1996*b*). However, this signalling hypothesis fails to account for reports of *Cah1* induction by low CO_2 in the dark, enhanced *Cah1* induction by non-photosynthetic blue light, and partial repression of *Cah1* by mixotrophic growth with acetate as a carbon source (Bailly and Coleman 1988; Dionisio *et al.* 1989*a*, *b*; Dionisio-Sese *et al.* 1990; Coleman *et al.* 1991; Fett and Coleman 1994; Rawat and Moroney 1995; Villarejo 1996*b*). Furthermore, in work with *Chlorella* species, Matsuda and Colman (1995, 1996) have reported results most consistent with the cells responding to the CO_2 concentration in the bulk medium, and Bozzo and Colman (2000) have reported similar results for *Chlamydomonas*. In addition, growth of *pmp1-1* in very low CO_2, but not in low CO_2 (Fig. 1), indicates multiple levels of acclimation to limiting CO_2. Clearly, acclimation of *C. reinhardtii* to limiting CO_2 involves a signalling system more complex than a change in the concentration of a photorespiratory metabolite but, at present, it is difficult to incorporate all the available information on acclimation into a cogent hypothesis for the signalling mechanism.

Not only is the nature of the limiting-CO_2 signal unclear, it is not even clear that there is a limiting-CO_2 signal. The real signal may occur in elevated CO_2, repressing the limiting-CO_2 acclimation response, raising intriguing parallels with other systems for metabolic regulation of gene expression, such as the glucose repression system in *Saccharomyces cerevisiae* (Gancedo 1998), where the presence of a preferred carbon source, glucose, represses transcription of genes required for catabolism of alternate carbon sources such as galactose. By analogy then, in *C. reinhardtii*, the presence of a rich carbon source, either elevated CO_2 or acetate, may repress the expression of genes necessary for growth in limiting CO_2. A repression signal in elevated CO_2, however, is difficult to reconcile with the apparent photosynthesis requirement for full acclimation or a blue-light enhancement of acclimation to limiting CO_2, unless limiting-CO_2 acclimation requires one or more induction signals along with absence of a repression signal, as in the expression of galactose catabolism genes in *S. cerevisiae* (Gancedo 1998). In addition, the potential for different levels of acclimation, as suggested by the growth response of the *pmp1-1* mutant, would make a simple 'rich carbon' repression system unlikely. It also is possible that there are parallel or interacting signal pathways involving induction and/or repression. Thus, much is yet to be learned about the signal or signals involved in the acclimation of *C. reinhardtii* to changes in CO_2 availability.

The identification of a mutant, *cia5-1* (Table 1), apparently incapable of any acclimation response to limiting CO_2 (Moroney *et al.* 1989), was an important advance in understanding gene regulation mediated by changes in CO_2 concentration. The absence of any known limiting-CO_2 acclimation responses in *cia5-1* (Moroney *et al.* 1989; Marek and Spalding 1991; Spalding *et al.* 1991) argues that all acclimation responses are regulated either by one signal transduction pathway, or by multiple pathways that all require the *Cia5* gene product. By analogy with yeast glucose repression, *cia5-1* is a de-repression mutant since, in the absence of sufficient carbon (elevated CO_2 or acetate), the *cia5* mutant is unable to de-repress the genes required for growth in limiting CO_2.

The *Cia5* gene was identified recently by complementation of the *cia5-1* mutant (Xiang *et al.* 2001). Almost simultaneously, Fukuzawa *et al.* (2001) identified the same gene, which they called *Ccm1*, as defective in another *cia5* allele (*cia5-2*; Table 1) generated by insertional mutagenesis. *Cia5* is a 6.5 kb gene composed of five introns and six exons (Fig. 2). The constitutively expressed 5.2 kb *Cia5* mRNA encodes a predicted 698 amino acid protein, which terminates near the 5′ end of the sixth exon, to leave a 3′ untranslated region of nearly 3 kb (Fig. 2). The predicted Cia5 protein has characteristics suggesting it may be a zinc finger-type transcription factor (Fukuzawa *et al.* 2001; Xiang *et al.* 2001), a suggestion supported by the mutation in *cia51* where a tyrosine residue is predicted to be substituted for one of the two histidine residues comprising

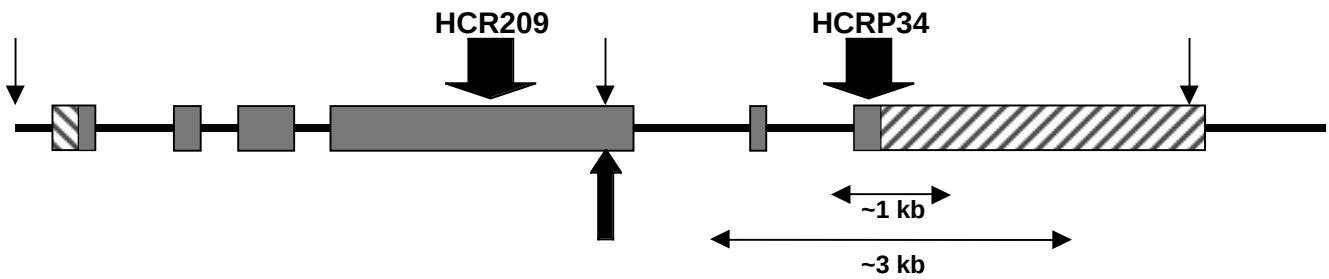

Fig. 2. Structure of the *Cia5* gene, showing its intron/exon configuration. The solid colour indicates the translated portions and the striped regions indicate the 5′ and 3′ untranslated regions of the exons. The lower, block arrow indicates the approximate site of truncation in the subgenomic complement of *cia5-1*, and the upper, block arrows indicate the approximate insertion sites of *Arg7* in mutants HCR209 and HCRP34. Modified from Xiang *et al.* (2001).

the first of two zinc finger motifs. Complementation of *cia5-1* with a subgenomic, 4.2 kb 5′ fragment of *Cia5* resulted in expression of a 2.1 kb mRNA encoding a slightly truncated Cia5 protein (Fig. 2). However, unlike in wild-type cells or in *cia5-1* cells complemented with an intact *Cia5* gene, the limiting-CO_2 induced genes *Cah1*, *Mca1*, and *Ccp2* were expressed constitutively in *cia5-1* cells complemented with the truncated Cia5 protein (Xiang *et al.* 2001). These results suggest that the deleted C-terminal domain allows, and is necessary for, Cia5 to mediate either the activation of these genes in limiting CO_2, or their repression in elevated CO_2.

Cia5 clearly is a key component in the regulation of limiting-CO_2 induced genes, and has characteristics consistent with a transcription factor. However, it is not clear whether it acts directly as a transcription repressor or activator of limiting-CO_2 regulated genes, directly on transcription of other repressors or activators, or in some indirect function as part of regulatory cascade. Below are described two of several potential models that are consistent with our current knowledge regarding Cia5 function.

Limiting CO_2 activation model

Cia5 activates transcription of limiting-CO_2 regulated genes, but only if not post-translationally modified in the *C*-terminal region. Post-translational modification of the *C*-terminal region in elevated CO_2 prevents Cia5 from activating expression of limiting-CO_2 regulated genes, so truncation results in a protein that constitutively activates their expression. In the absence of a functional Cia5, transcription of the limiting-CO_2 regulated genes is not activated.

Elevated CO_2 repression model

Cia5 acts as an upstream regulator that prevents expression of, or otherwise inactivates, a repressor of limiting-CO_2 regulated gene transcription, but only if not post-translationally modified in the *C*-terminal region. Post-translational modification of the *C*-terminal region in elevated CO_2 prevents inactivation of the repressor, so limiting-CO_2 regulated genes are not expressed in elevated CO_2. Truncation of Cia5 results in a protein that constitutively inactivates the repressor, resulting in constitutive expression of limiting-CO_2 regulated genes. In the absence of a functional Cia5, the repressor cannot be inactivated, so limiting-CO_2 regulated genes are constitutively repressed.

New insertional mutants

Identification of *cia5-1*, and of the *Cia5* gene, has provided some insight into the regulation of limiting-CO_2 acclimation (Moroney *et al.* 1989; Fukuzawa *et al.* 2001; Xiang *et al.* 2001). Although additional mutants would be valuable in understanding this signal transduction pathway, few other signal transduction mutants have been reported. As clearly illustrated by its use in the cloning of *Ccm1* (Fukuzawa *et al.* 2001) and by the number of other researchers generating insertional mutants for this purpose (Colombo *et al.* 2002; Miura *et al.* 2002; Thyssen *et al.* 2002), the application of insertional mutagenesis represents a powerful approach to analysis of the acclimation response of *C. reinhardtii* to changes in CO_2 availability.

Putative new *C. reinhardtii* de-repression mutants were identified using *Cah1* expression as a reporter for limiting-CO_2 acclimation. A cell wall-less *arg2* mutant (strain CC425; *arg2 cw15 sr-u-2-60 mt+*) was complemented by transformation (Kindle 1990) with an *Arg7*-containing plasmid (pARG7.8, Debuchy *et al.* 1989; or pJD67, Davies *et al.* 1994) to generate putative de-repression mutants. From several thousand arg+ colonies, 19 independent, putative de-repression mutants (Table 3) were selected through screens either involving replica plating in elevated CO_2 (5% CO_2 in air), low CO_2 (normal air), and very low CO_2 (<100 μL L^{-1} CO_2) to identify HCR mutants (Van *et al.* 2001), or involving immunoslot blots and western immunoblots to identify mutants in which pCA1 abundance was decreased or absent (Van and Spalding 1999).

Growth tests in high CO_2, low CO_2 and very low CO_2 conditions confirmed a HCR phenotype in 13 mutants, nine of which had stringent (no growth either in low CO_2 or very low CO_2) and four of which had leaky (slow growth in very low CO_2) HCR phenotypes. Western immunoblots for pCA1 and other limiting-CO_2 induced proteins found various patterns of decreased abundance among the mutants (Table 3). However, based on RNA blots with the limiting-CO_2 regulated genes *Cah1*, *Mca1* and *Ccp1*, only 57-61, HCRP34 and HCR209 lacked *Cah1* mRNA completely, and only HCRP34 and HCR209 lacked all three limiting-CO_2 regulated messages in low CO_2 (Table 3). Three other mutants, HCR90, HCR95 and HCR99, had reduced abundance of one or more of the messages. HCR90 had reduced expression of only *Mca1* and *Mca2* mRNA (Van *et al.* 2001), HCR99 had reduced expression of all three, and HCR95 showed only a transient induction of these three genes (Van *et al.* 2001; Table 3). HCR99 may have a general defect in transcription or RNA stability, because message abundance was decreased similarly for several other, unrelated genes. The other HCR mutants (HCR3510, HCR86, HCR89 and HCR105) did not show reproducibly different patterns of expression for the three limiting-CO_2 regulated mRNAs.

Eleven of the mutants were subjected to genetic analysis (Van and Spalding 1999; Van *et al.* 2001; Table 4). Nine of these contain only one copy of the *Arg7* insert, and the inserts co-segregated with the mutant phenotype in six mutants, indicating that the *Arg7* insert probably is responsible for their phenotypes. In two mutants, HCR95 and HCR105, the inserts and phenotypes did not co-segregate, indicating that the insert was not directly responsible for their phenotypes. In HCR209 and HCR103, which have two

and three inserts, respectively, co-segregation crosses were not conclusive. Other evidence (see below) suggests the two inserts in HCR209 are tandemly arranged, and are responsible for the phenotype. Because of a probable general defect in transcription, HCR99 was not tested further.

Construction of heterozygous vegetative diploids (Van *et al.* 2001) confirmed that all 12 mutants had recessive phenotypes (Table 4). Rapid allelism tests were used to place eight of the HCR mutants, and the previously described HCR mutants *cia5-1*, *ca1-1*, *pmp1-1* and *pgp1-1*, into complementation groups (Van *et al.* 2001; Table 5). Only crosses *cia5-1* × HCRP34, *cia5-1* × HCR209 and HCRP34 × HCR209 failed to generate wild-type colonies, indicating that HCR3510, HCR86, HCR89, HCR90, HCR95 and HCR105 each define a new HCR locus.

DNA sequences flanking the inserts were obtained for six of the insertional mutants (Table 4). The insert in 57-61 disrupted *Cah1*, facilitating an investigation of the contribution of pCA1 to limiting-CO_2 acclimation in *C. reinhardtii* (Van and Spalding 1999). DNA flanking both sides of the insert in HCR89 was cloned and sequenced. It disrupted a D-lactate dehydrogenase/glycolate dehydrogenase gene via a simple insertion in the first intron of the gene. A paper describing this work will be published elsewhere (Y. Nakamura, S. Kanakagiri, K. Van and M. Spalding, unpublished results).

Genetic analyses indicated that both HCRP34 and HCR209 are allelic to *cia5-1*. DNA flanking both sides of the insert in HCRP34 and one end of the two tandemly arranged inserts in HCR209, was cloned and sequenced. Comparison with a cloned *Cia5* gene (Xiang *et al.* 2001) established that the *Arg7* insert in HCRP34 disrupted *Cia5* via a simple insertion in the sixth exon just upstream from the termination codon, and that the inserts in HCR209 disrupted *Cia5* in the fourth exon (Fig. 2). HCRP34 and HCR209 were confirmed as defective in the CIA5 locus by complementation (data not shown) with *Cia5* (Xiang *et al.* 2001).

DNA flanking both sides of the inserts in HCR86 and HCR90 was cloned and sequenced. Three BAC clones were identified that hybridize to flanking regions from HCR86, and all were demonstrated to complement HCR86. Identification and characterization of six BAC clones that hybridize to the flanking regions of HCR90, as well as several additional BAC clones overlapping the original six, indicated a large disruption had occurred at the insertion site in this mutant. None of the selected BACs have complemented HCR90. Identification of the genes defective in these two mutants is continuing.

Table 3. General characteristics of insertional mutants

WT, similar to wild-type growth; LHCR, growth much slower than WT; SHCR, no growth; LC, low CO_2 (~300–500 μL^{-1} CO_2); VLC, very low CO_2 (~50–100 $\mu L\ L^{-1}$ CO_2). Western blots with specific antisera were used to determine the presence of pCA1, Ccp (LIP36) and mtCA (LIP21), and mRNA levels for the three genes were determined with northern blots normalized to ribosomal RNA. +, approximately normal protein or mRNA level; ±, less than 50% of normal protein or mRNA level; –, no protein or mRNA detectable; ND, not determined

	Phenotype		*Cah1*		*Ccp1/2*		*Mca1/2*	
Strain	LC	VLC	pCA1	mRNA	Ccp	mRNA	mtCA	mRNA
CC425	WT	WT	+	+	+	+	+	+
ca1-1	LHCR	SHCR	+	+	+	+	+	+
pmp1-1	SHCR	WT	+	+	ND	+	ND	+
cia5-1	LHCR	SHCR	–	–	–	–	–	–
HCRP34	LHCR	SHCR	ND	–	ND	–	ND	–
HCR209	LHCR	SHCR	ND	–	ND	–	ND	–
HCR3510	WT	SHCR	ND	+	ND	+	ND	+
HCR86	SHCR	SHCR	±	+	+	+	–	+
HCR89	LHCR	LHCR	ND	+	ND	+	ND	+
HCR90	LHCR	SHCR	±	+	±	+	–	±
HCR95	LHCR	LHCR	±	±	–	±	+	±
HCR99	LHCR	LHCR	±	±	–	±	+	±
HCR103	LHCR	LHCR	±	+	–	+	+	+
HCR105	SHCR	SHCR	ND	+	ND	+	ND	+
2–29	WT	WT	±	ND	+	ND	+	ND
24–39	WT	WT	±	ND	±	ND	+	ND
25–08	WT	WT	±	±	±	±	+	±
29–08	WT	WT	±	±	+	±	+	±
34–29	WT	WT	±	±	+	±	±	±
40–89	WT	WT	±	ND	+	ND	+	ND
57–61	WT	WT	–	–	+	+	+	+
63–24	WT	WT	±	ND	+	ND	+	ND
66–18	WT	WT	±	ND	±	ND	±	ND

Table 4. Genetic characteristics of insertional mutants
NA, not applicable; ND, not determined. Table modified from Van *et al.* (2001)

Strain	Defective gene	Insert copy number	Co-segregation with insert	Flanking DNA	Diploid analysis
CC425	NA	NA	NA	NA	—
ca1-1	*Cah3*	NA	NA	NA	Recessive
pmp1-1	?	NA	NA	NA	Recessive
cia5-1	*Cia5*	NA	NA	NA	Recessive
HCRP34	*Cia5*	1	Yes	Yes	Recessive
HCR209	*Cia5*	2	?	Yes	Recessive
HCR3510	?	1	Yes	No	Recessive
HCR86	?	1	Yes	Yes	Recessive
HCR89	*Gdh1*	1	Yes	Yes	Recessive
HCR90	?	1	Yes	Yes	Recessive
HCR95	?	1	No	No	Recessive
HCR99	?	1	ND	No	Recessive
HCR103	?	3	?	No	Recessive
HCR105	?	1	No	No	Recessive
57–61	*Cah1*	1	Yes	Yes	Recessive

Implications of new mutants for signal transduction

Some of the new insertional mutants discussed above, such as 57-61 (*cah1-1*; Table 1) and HCR89 (*gdh1-1*; Table 1), clearly are not directly involved in the signal transduction pathway for acclimation of *C. reinhardtii* to changes in CO_2 availability. The two new alleles of cia5, HCRP34 (*cia5-3*; Table 1) and HCR209 (*cia5-4*; Table 1), clearly are involved, and the two mutants with altered expression of one or more limiting-CO_2 regulated genes, HCR90 and HCR95, are good candidates for signal transduction mutants. The phenotype of HCR95 suggests there may be separable pathways for transient and sustained responses, and the phenotype of HCR90 suggests there may be specific factors involved in regulation of individual genes, in addition to general factors like *Cia5*. The other characterized HCR mutants, HCR3510, HCR86, HCR103 and HCR105, would seem to be less likely candidates for defects in signal transduction, because no altered expression of the limiting-CO_2 regulated genes was observed. These mutants, especially HCR86 and HCR105, which do not grow in either low or very low CO_2, probably represent better candidates for lesions in CCM functional components. However, it is unlikely that expression of the three limiting-CO_2 regulated genes used as reporters here is sensitive to all defects in the signal transduction pathway, so one or more of these mutants could affect a part of the signal transduction pathway unrelated to these genes. If the differing growth responses of *pmp1-1* at low CO_2 and very low CO_2 concentrations (see above) truly represent different levels of acclimation, then a mutant such as HCR3510, which grows like wild-type in low CO_2, but not at all in very low CO_2, becomes very interesting. This is a characteristic one might expect of a mutant specifically deficient in acclimation to very low CO_2, or with a lesion in the Ci transport induced specifically in very low CO_2. The non-HCR mutants, other than 57-61, have proven difficult to analyse further because they lack a visible phenotype and have high variability in message abundance.

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